OFFICE OF NAVAL RESEARCH

CONTRACT N00014-88-C-0118
CONTRACT N00014-94-C-0149

TECHNICAL REPORT 95-07

THE EFFECT OF HEPARIN ON FIBRINOLYTIC ACTIVITY AND PLATELET FUNCTION

BY

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16 SEPTEMBER 1995

Abstract

Heparin, a polyanionic glycosaminoglycan, is used routinely prior to the induction of cardiopulmonary bypass. Earlier observations in our laboratory suggested that the postoperative bleeding which occurs, despite neutralization of heparin with protamine, is secondary to hypothermia and dilutional anemia during bypass. An additional, potential mechanism for excessive bleeding following cardiopulmonary bypass is that heparin activates the fibrinolytic system, which may, in turn, adversely affect hemostasis. To understand better the effects of heparin administration on the fibrinolytic system *in vivo*, we simulated the anticoagulant regimen of cardiopulmonary bypass by administering increasing doses of intravenous heparin to five adult baboons over 60 minutes. We measured fibrinolytic parameters serially following heparinization, and demonstrated that heparin induces activation of the fibrinolytic system. We showed that the fibrinolytic system was activated *in vivo* as evidenced by an increase in plasmin activity and immunoreactive plasmin light chain, as well as an increase in immunoreactive fibrinogen fragment E in vitro. These results demonstrate that the fibrinolytic system is activated *in vivo* by the administration of heparin during cardiopulmonary bypass. These data suggest that, despite administration of a neutralizing agent such as protamine, heparin may contribute to postoperative bleeding complications following cardiopulmonary bypass surgery owing principally to its longer-lived effects on the fibrinolytic system.

Introduction

The generalized hemorrhagic tendency accompanying cardiopulmonary bypass has been recognized for over a decade. An abnormality in platelet function is believed to be the principal hemostatic defect underlying this hemorrhagic tendency (16,42,17,7); however, abnormalities in the coagulation and fibrinolytic systems have also been reported (26,22). We have recently attempted to characterize the specific defect in platelet function that accompanies cardiopulmonary bypass, and concluded that the defect is extrinsic to the platelet (21); intrinsic platelet function appears to be preserved.

The high circulating concentrations of heparin used during cardiopulmonary bypass are likely to account in part for platelet dysfunction. Inactivation of circulating thrombin, the most important platelet agonist *in vivo* (15,8,20), by antithrombin III is dependent upon heparin. In addition, the relative protection of thrombin from antithrombin III afforded by fibrin binding is overcome by the high concentrations of heparin used during cardiopulmonary bypass (40) [4.5 U/ml with an activated clotting time $\geq$ 999 seconds in our original study (21)]. Coupled with the direct inhibitory effects of hypothermia on platelet activation (21,36,28), the inhibition of thrombin by heparin provides a rational mechanism for attenuated platelet responses during bypass.

Heparin, however, has a number of profibrinolytic effects that have

been appreciated for many years which could also contribute to platelet dysfunction (14,10,35,9). Mechanisms by which this polydispersed glycosaminoglycan is believed to facilitate fibrinolysis are several and include direct stimulation of the release of plasminogen activator(s) from vascular (27) and monocytic cells; facilitation of the activation of protein C, promoting the release of plasminogen activator; impairment of fibrin monomer polymerization; enhancement of the catalytic efficiency of plasminogen activation by urokinase-type plasminogen activators and tissue-type plasminogen activator enhancement of plasmin-mediated activation of single-chain urokinase-type plasminogen activator; modulation of antiplasmin activities (12,2); catalysis of thrombin-induced neutralization of plasminogen activator inhibitor type 1; and potentiation of the effects of tissue factor pathway inhibitor, thereby reducing the modulatory prothrombotic effects that often accompany fibrinolysis. These *in vitro* observations, coupled with the recent reports on the *in vivo* benefits of aprotinin on blood loss during cardiopulmonary bypass (39,37), suggest that heparin use may underlie the platelet dysfunction of cardiopulmonary bypass both by impairing thrombin activity and promoting the development of a fibrinolytic milieu in which platelets are rendered additionally dysfunctional.

In this report, we show simulated the anticoagulant regimen of cardiopulmonary bypass in normal baboons to show that these doses (10, 100, 400, and 500 U/kg) that heparin leads to plasminogen activation *in vivo*;

that the generation of plasmin is accompanied by evidence for its systemic lytic effects; and that platelets exposed to heparin *in vivo* are more susceptible to activation as determined by whole blood flow cytometry. The enhancement of the activation of plasminogen by heparin and the subsequent generation of plasmin likely contributes to the hemostatic defect of cardiopulmonary bypass.

Materials and Methods

Animal Protocol

This study was conducted on five male baboons, aged 15-25 years, at the Naval Blood Research Laboratory, Boston University School of Medicine, Boston, MA. Animal studies were performed in accordance with institutional Animal Care Committee regulations. Table 1 reports the hematologic and coagulation parameters in five baboons weighing 27.6 ± 1.0 kg prior to and following heparin administration. The design of this study was based on a progressive weight-adjusted increase in intravenous heparin administration. Heparin was given after baseline laboratory studies were obtained at t = 0, and increasing doses were administered at 10, 20, 40 and 60 min (heparin doses = 10, 100, 400, and 500 U/kg, respectively). Ten minutes after each of the heparin doses was administered, blood samples were collected. Heparin (Abbott Laboratories, Abbott Park, IL) was neutralized at the end of the study period with protamine sulfate (Abbott Laboratories, Abbott Park, IL) (mean 118.8 ± 37.5 mg, range 75–150 mg), and the efficacy of reversal was monitored by the measurement of the activated clotting time.

Blood Specimens: Acquisition and Assays

Blood was collected into tripotassium ethylenediaminetetraacetic acid (EDTA) for the measurement of hematocrit and platelet count using a Coulter JT counter (Coulter Electronics, Hialeah, FL). Whole blood was collected into

trisodium citrate and centrifuged at 1000 x g for 10 min, and plasma samples were stored at –70°C until all samples could be assayed simultaneously.

Samples for the measurement of fibrinogen were collected in 3.8% trisodium citrate and fibrinogen concentrations were measured with a Coag–a–Mate X–2 photo-optical instrument (Organon Teknika Corp., Durham, NC). Heparin concentrations were measured using a chromogenic substrate (22). D–dimer, a fibrinolytic degradation product of fibrin, was measured by an enzyme–linked immunosorbent assay (ELISA) with a specific monoclonal antibody (22). The activated partial thromboplastin time was measured according to standard methods used in our laboratories (22). Plasmin activity in plasma was determined as previously described using the chromogenic substrate S2251 and diluted plasmin and is expressed in U/L (22). Bleeding time was measured from the lateral aspect of the volar surface of the forearm according to the method of Babson and Babson (3) with the Simplate II bleeding time device (Organon Teknika Corp., Durham, NC). All bleeding times were performed in duplicate in a room with a constant ambient temperature (36).

Western Blot Assays

The conversion of plasminogen to plasmin by activation of the fibrinolytic system was determined using Western blotting techniques. Plasma anticoagulated with 3.8% trisodium citrate was electrophoresed on a 7-15%

gradient polyacrylamide gel under reducing conditions. The gel was transferred onto a polyvinylidene difluoride (PVDF) membrane and the membrane incubated with a goat polyclonal antihuman plasminogen antibody that cross-reacted with baboon plasminogen. Plasminogen was identified by its mobility and quantified by densitometry (30).

The *in vivo* activity of plasmin was determined by measuring selected fibrinogen degradation products using Western blotting techniques, as well. Plasma anticoagulated with 3.8% trisodium citrate was electrophoresed on a 7-15% gradient polyacrylamide gel under nonreducing conditions. The gel was transferred onto a PVDF membrane and the membrane was incubated with a goat polyclonal antihuman fibrinogen antibody that cross-reacted with baboon fibrinogen. Fragment E was identified by its mobility and quantified by densitometry (30).

Murine Monoclonal Antibodies

S12 (provided by Dr. Rodger P. McEver, University of Oklahoma) is a monoclonal antibody directed against human P-selectin that cross-reacts with baboon P-selectin (34,4). P-selectin, also referred to as GMP-140 (34), platelet activation-dependent granule-external membrane (PADGEM) protein (18), and CD62P (33), is a component of the α granule membrane of resting platelets that is only expressed on the platelet plasma membrane after platelet secretion (34).

TM60 (provided by Dr. Naomasa Yamamoto, Tokyo Metropolitan Institute of Medical Science) is directed against the thrombin binding site on the amino-terminal domain of the α chain of human GPIb that cross-reacts with baboon GPIb (19). Y2/51 (Dako, Carpinteria, CA) is directed against platelet membrane GPIIIa (11).

Measurement of Platelet Surface Glycoproteins

A whole blood flow cytometric method was used, as previously described (21). The method includes no centrifugation, gel filtration, vortexing, or stirring steps that could artifactually activate platelets. The first 2 ml of baboon blood drawn were discarded and then blood was drawn into a Vacutainer (Becton Dickinson, Rutherford, NJ) containing trisodium citrate and, within 15 minutes, diluted in modified Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 0.35% bovine serum albumin, 10 mM HEPES, 5.5 mM glucose, pH. 7.4). The sample was then incubated (22°C, 15 min) with a combination of 1 μM adenosine 5'-diphosphate (ADP) (Bio Data, Hatboro, PA) and 5 μM epinephrine (Sigma, St. Louis, MO), or buffer only. The samples were then fixed at 22°C for 30 min with formaldehyde (1% final concentration), diluted, and incubated (22°C, 15 min) with a saturating concentration of a biotinylated monoclonal antibody (S12 or TM60) and a near saturating concentration of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody Y2/51. The

samples were subsequently incubated at 22°C for 15 min with phycoerythrin-streptavidin (Jackson Immuno-Research, West Grove, PA). Within 24 hr of antibody tagging, the samples were analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL). After identification of platelets by gating on both FITC positivity and their characteristic light scatter, binding of the biotinylated monoclonal antibody was determined by analyzing 5,000 individual platelets for phycoerythrin fluorescence. Results are presented as a percent of maximal expression of sulfate antigen in response to the potent agonist thrombin, as previously described (21).

Data Analysis

Comparisons were made between data obtained before and after the administration of heparin using a paired Student's t–test. Values presented represent the mean ± S.E.M., and p values less than 0.05 were considered statistically significant.

Results

Calculated Heparin Dose and Serum Heparin Level

Heparin was administered to stimulate levels achieved during corresponding bypass as described in Materials and Methods based on the body weight of the animals in kilograms. At 10, 20, 40, and 60 mins, heparin was administered intravenously and cumulatively at 10, 100, 400, and 500 U/kg body weight. Ten minutes following heparin administration (20, 30, 50, and 70 min), blood was drawn for determination of heparin concentrations. Actual plasma heparin concentrations were found to be 0.20 $\pm$ 0.01 U/ml, 1.32 $\pm$ 0.07 U/ml, 7.80 $\pm$ 0.13 U/ml, and 13.00 $\pm$ 1.10 U/ml compared to a baseline plasma heparin level of <0.20 U/ml ($p<0.001$ compared to 30, 50, and 70 mins).

Effect of Heparin on Fibrinolysis

Figure 1 demonstrates the correlation between plasmin activity and plasmin light chain levels *in vivo* as determined by densitometric quantification of Western blots of baboon plasma. Linear regression analysis shows a correlation coefficient between plasmin activity and plasmin light chain content of $R=0.65$ ($p=0.010$). The modest quality of the goodness-of-fit of this analysis suggests the possibility that a threshold level of plasmin light chain must be achieved to result in measurable plasmin activity in these plasma samples not derived of serpins.

Having demonstrated a linear correlation between plasmin activity and plasmin light chain generation, we next examined the effects of increasing heparin concentrations added *in vitro* on plasmin light chain in baboon plasma. Heparin, from 0.1 to 10 U/ml, was added to plasma, and plasmin light chain was quantified. Plasmin light chain increased following incubation of plasma with heparin at concentrations of 1 U/ml to 10 U/ml. Figure 2 is a scatter plot that demonstrates a linear increase in plasmin light chain as the concentration of heparin increases; linear regression analysis shows a correlation coefficient of $R=0.71$ ($p=0.012$). Again, the modest quality of the goodness-of-fit of these data suggest that the need to achieve a threshold level of heparin for measurable plasmin activity.

In vivo studies in baboons showed that increasing heparin doses cause a time-dependent increase in plasmin activity. Following administration of 100 U/kg heparin (30 min), plasmin light chain increased significantly compared with baseline values (0.58 ± 0.29 U/L at baseline compared to 1.40 ± 0.72 U/L at 30 min, $p=0.02$) (Figure 3). Plasmin activity was maximally stimulated by 50 min compared to baseline, (72.70 ± 6.40 U/L, $p=0.0003$) and returned to baseline 24 hrs following administration of heparin (1.04 ± 0.54 U/L at 24 hours).

Other markers of fibrinolysis examined over time following heparin administration included plasma levels of fibrinogen and D–dimer. Fibrinogen levels decreased 20 min after administration of heparin (180 $\pm$10 mg/dl at

baseline compared to 173 ± 9 mg/dl at 20 mins) and remained decreased for 70 min (158 ± 8 mg/dl), although the magnitude of the decrease did not reach statistical significance ($p=0.67$) (Table 1). Twenty-four hours following the initial administration of heparin, fibrinogen levels demonstrated a rebound increase maximally to 272 ± 23 mg/dl, ($p<0.004$ compared to control).

Concurrent with the increase in plasmin light chain, a rise in D–dimer occurred following heparinization of the baboons (Table 1). D-dimer tended to increase from 123 ± 41 ng/ml at baseline to 140 ± 48 ng/ml at 20 min ($p = 0.09$) and tended to remain elevated 24 hrs following heparin administration (441 ± 152 ng/ml, $p = 0.14$)

Having demonstrated a near significant increase in D-dimer level following heparin administration to baboons, we next examined the effect of increasing heparin concentration on a specific fibrinogen degradation product (FDP), fragment E. Using quantitative Western blotting techniques, we found a concentration-dependent increase in fragment E generation when heparin is added *in vitro* to baboon plasma (Figure 4). Linear regression analysis shows a correlation coefficient between fragment E and heparin concentrations of $R=0.89$ ($p=0.0067$).

Effect of Heparin on Hematologic and Coagulation Parameters

Platelet count, hematocrit, bleeding time, clotting time, and activated partial thromboplastin time (APTT) were measured at baseline, 20, 30, 50, and

70 min, as well as 24 hrs after heparin administration. These measurements are reported in Table 1. The data demonstrate that hematocrit decreases 24 hrs following heparin administration compared to baseline values. Simultaneously, a decrease in platelet count occurs reaching a nadir at 70 min following heparin administration. This decrease in platelet count is paralleled by an increase in bleeding time at 70 min, as well as an increase in clotting time beginning 30 min following initial heparin administration and continuing until completion of the study. No change in APTT was demonstrated 24 hrs following heparin administration, while no clot formed (> 100 sec) at 30, 50 or 70 min.

Effect of Heparin on Platelet Activation

Using flow cytometry of platelets obtained from baboons treated with heparin, we observed significant changes in P-selectin and glycoprotein Ib markers as a function of heparin dose administered *in vivo*. At concentrations greater than 1U/ml, heparin augments the ADP- and epinephrine-induced increase in platelet-surface P-selectin (S12) (Figure 5A) and concomitant decrease in platelet-surface glycoprotein Ib (TM60) (Figure 5B). As shown by parallel experiments in the absence of exogenous agonists, heparin administration itself results in neither platelet degranulation, as determined by lack of expression of platelet-surface P-selectin (Figure 5A), nor platelet activation as determined by the lack of decrease in platelet surface glycoprotein Ib (Figure 5B).

DISCUSSION

Heparin has been used since the institution of cardiopulmonary bypass to prevent thrombotic complications. This polyanionic glycosaminoglycan elicits its anticoagulant effect by binding to antithrombin III and catalyzing the interaction of this serpin with procoagulants, including thrombin (15,8,20). While the administration of protamine following cardiopulmonary bypass neutralizes heparin's anticoagulant activity, recent studies suggest that heparin also has a profound effect on fibrinolysis and platelet function (26,22,21,23,24). Therefore, the present study was undertaken in an attempt to understand better the effect of isolated heparin administration on fibrinolysis and platelet function in a primate model.

This study demonstrates that the administration of heparin causes an increase in fibrinolytic activity *in vivo*, as well as an increase in the susceptibility of platelets to activation. While heparin administration has been shown to cause an increase in plasmin activity prior to cardiopulmonary bypass in patients, direct *in vivo* studies of plasminogen conversion have been lacking (9). In the present study, we first demonstrated a linear correlation between plasmin activity and plasmin light chain generation using Western techniques. Following this observation, plasma from baboons, to which increasing doses of heparin were administered, showed an increase in plasmin light chain as heparin concentration increased. In addition, using Western techniques we showed a significant increase in a specific FDP, fragment E,

with increasing heparin concentration. This rise in fragment E following heparin addition to plasma *in vitro* suggests that FDP may play a role in causing platelet dysfunction *in vivo* as FDP have been shown to interfere with platelet aggregation by binding to glycoprotein IIb/IIIa; these largely monovalent ligands compete with bivalent fibrinogen and thereby inhibit platelet-platelet interactions (25).

Simultaneously, *ex vivo* studies using flow cytometry showed that platelets stimulated with subthreshold concentrations of ADP and epinephrine expressed more P-selectin and less glycoprotein Ib with increasing heparin doses administered *in vivo*. These data suggest that heparin renders platelets more susceptible to activation by agonists *in vitro*. Despite this enhanced activation response, profibrinolytic and hypothermic effects combined may tend to promote nonsurgical bleeding in patients treated with heparin or bypass. The present study confirms an earlier observation by Kestin and colleagues which suggested that heparin has at least two distinct effects on platelet activation/function (21). *In vitro* heparin augments platelet activation; yet *in vivo* by inhibiting endogenous thrombin, heparin paradoxically suppresses platelet activation.

It has been recognized for over a decade that heparin used during cardiopulmonary bypass can contribute to the development of an hemorrhagic diathesis by a number of different mechanisms. Fareed first reported an increase in t-PA levels in normal volunteers after the administration of both

subcutaneous and intravenous heparin (9). Subsequent studies by Giuliani and colleagues further proved that heparin was profibrinolytic when they showed that D–dimer was increased during cardiopulmonary bypass (13). Basic studies at the molecular and cellular level have been lacking, but a recent study showed that heparin (and heparin–like compounds) is capable of inducing an increase of 10- to 17-fold in cell-surface plasminogen activation (31).

As heparin generates a profibrinolytic state, plasmin antigen levels, as well as activity, are increased. This rise in plasma levels of plasmin may lead to cleavage of GPIb or to proteolysis of von Willebrand proteins as suggested by *in vitro* studies (1). The importance of reversing the fibrinolysis that occurs with cardiopulmonary bypass and heparin administration has been underscored by studies in which bleeding tendencies were reversed or at least decreased using plasmin inhibitors such as aprotinin (6,5) or ϵ-aminocaproic acid (38). For example, aprotinin administration prior to bypass has been shown to prevent the formation of FDP and the consumption of α_2–antiplasmin activity (6). Others have demonstrated a significant decrease in blood loss, as well as a decrease in non–donor directed blood use, following the administration of aprotinin prior to cardiopulmonary bypass (5). This *in vivo* effect of aprotinin has been postulated to be secondary to aprotinin's ability to inhibit the plasmin–induced cleavage of GPIb (39), although we were unable to substantiate this mechanism in earlier studies

(21,29).

It has been suspected that the hemorrhagic diathesis following cardiopulmonary bypass may in part also be secondary to bypass-induced alteration in platelet function. Speculation initially focused on the effects of heparin on specific glycoproteins on the platelet surface. Loss of platelet receptors GPIb and GPIIb/IIIa have been reported following the institution of cardiopulmonary bypass (39,32,41). Yet, these initial studies used preparation techniques (fixed and washed platelets) that have led investigators to doubt the *in vivo* validity of these findings. Recently, however, using a whole blood flow cytometric method, Kestin and colleagues demonstrated in 20 patients undergoing cardiopulmonary bypass that neither the GPIb/IX complex nor the GPIIb/IIIa complex were decreased (21). These authors suggested that heparin suppressed platelet activation by inhibiting endogenous thrombin, an important *in vivo* platelet agonist.

The present study helps to explain heparin's direct effect on platelet function following cardiopulmonary bypass. Interventions aimed at reducing fibrinolysis and subsequent platelet dysfunction are important tools for the cardiovascular surgeon operating on patients who are especially prone to bleeding, such as those undergoing reoperation. Interventions, such as the administration of aprotinin prior to cardiopulmonary bypass or the use of lower activated clotting times, may become standard practice in patients considered to be at high risk for postoperative bleeding. Direct inhibitors of

thrombin, such as hirudin and hirulog, may also prove to produce less hemorrhage than traditional heparin used during bypass, owing principally to their lack of effect on the fibrinolytic system. Ongoing and future studies of patients undergoing cardiopulmonary bypass will need to address this important issue.

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Table 1 Hematologic and Coagulation Parameters in five Baboons Priot to and Following Heparin Administration

Parameter	Baseline	20 min	30 min	50 min	70 min	60 min post-protamine	24 hrs
Fibrinogen (mg/dl)	180±10	173±9	172±5	165±10	158±8	183±8	272±23*
Plasmin activity (U/L)	0.58±0.29	0.41±0.15	1.40 ±0.33*	72.7±6.40**	57.6±2.04**	1.22±0.31*	1.04±0.54
D-Dimer (ng/ml)	123±41	140±48	138±49	136±42	131±38	127±44	441±152
Hct (%)	38.6±1.2	38.8±0.8	39.2±1.2	38.7±1.0	38.9±1.0	39.1±1.0	36.9±1.4*
Platelet (x1000/μl)	251±17	245±17	246±15	221±19	211±15*	265±28	252±27
Bleeding time (min)	3.1±0.1	3.5±0.0*	4.0±0.2*	4.3±0.2**	4.8±0.3**	3.8±0.1*	3.2±0.1
Clotting time (sec)	195±34	357±63*	>1500*	>1500*	>1500*	262±41*	217±23
APTT (sec)	42.4±5.2	87±21	no clot**	no clot**	no clot**	86±22	86±27

All values are given as mean ±SEM for N=5 baboons. * $p<0.05$ compared to baseline, ** $p<0.001$ compared to baseline.

LEGENDS

Figure 1. Linear correlation of plasmin activity with plasmin light chain generation *in vivo*. Baboon plasma plasmin activity measured spectrophotometrically as U/L was plotted vs. plasma plasmin light chain content from Western blots quantified in arbitrary densitometric units. Each baboon plasma specimen ($N=5$) was analyzed at 50 minutes, two at 70 minutes, and one at 30 minutes. Results indicate that a linear correlation exists between plasmin activity and plasmin light chain with $R=0.65$ ($p<0.01$).

Figure 2. Effect of increasing doses of heparin on plasmin light chain generation *in vitro*. This scatter plot, showing plasmin light chain content following incubation of plasma with heparin (0.1-10 U/ml), demonstrates an increase in plasmin light chain as the concentration of heparin increases.

Figure 3. Effect of a progressive increase in heparin dosage on plasmin activity *in vivo*. Increasing heparin concentration over time causes an increase in plasmin light chain from 30 min until 70 min. Data are presented as mean $\pm$ SEM ($^{*}p<0.02$ and $^{**}p<0.0003$).

Figure 4. Effect of increasing heparin concentrations on fragment E in baboon plasma. Fragment E was measured by Western techniques following

incubation of plasma from two baboons with heparin *in vitro* and shows a significant increase as a function of increasing heparin concentration ($R=0.89$, $p=0.0067$). Each point represents the average of two experiments using two different baboon plasmas.

Figure 5. Effect of increasing heparin dose administered to baboons *in vivo* on platelet activation *in vitro* using whole blood flow cytometric analysis. Addition of subthreshold concentrations of ADP (1 μM) and epinephrine (5 μM) (closed squares) was accompanied by an increase in S12 binding (P-selectin) (A) and a decrease in TM60 binding (GPIb) (B) at concentrations of heparin greater than 1 U/ml compared with control (closed circles) ($N=4$).

Figure 1, Upchurch, et al.
Ms. # H0829-5

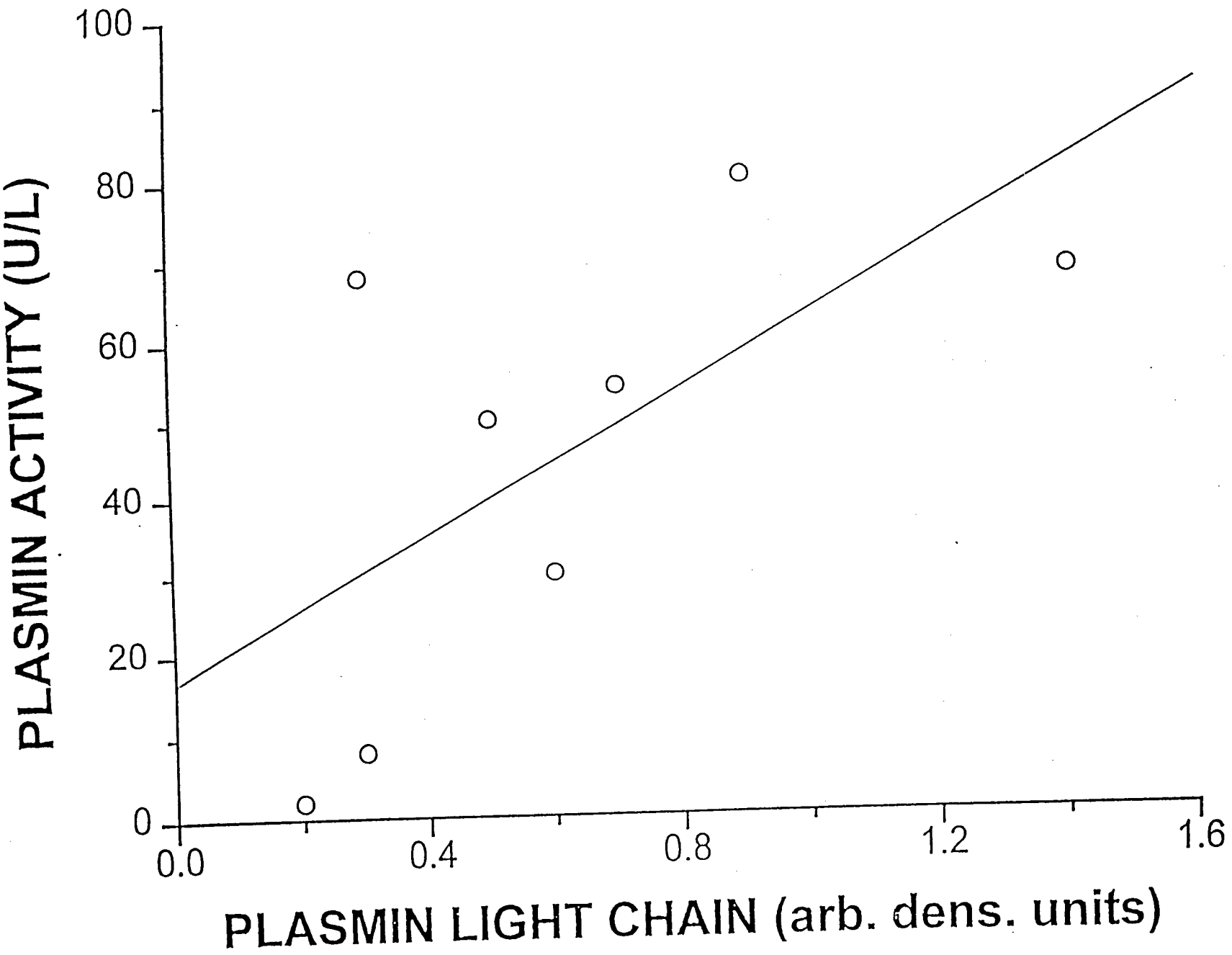

Figure 2, Upchurch, et al.
Ms. # H0829-5

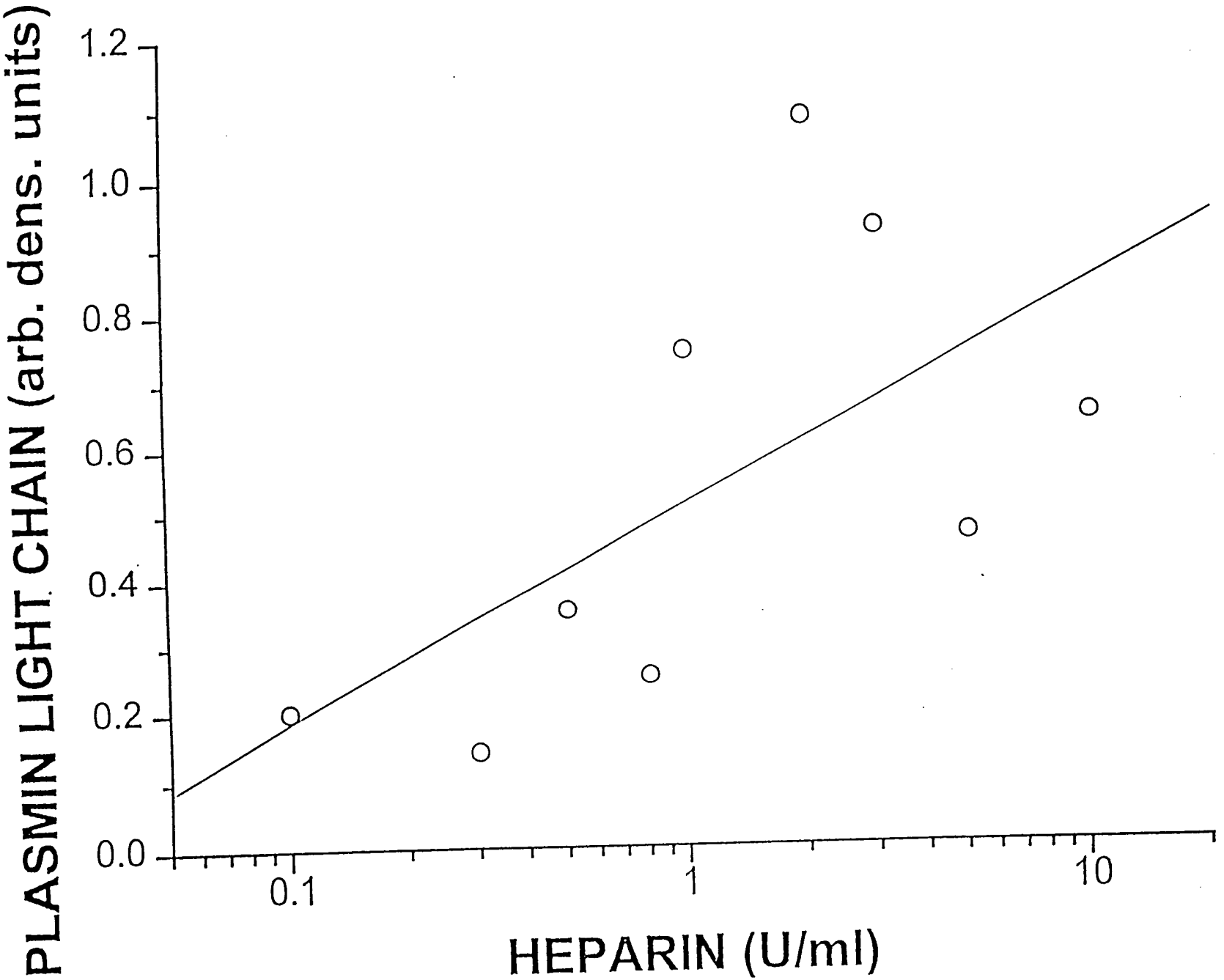

Figure 3, Upchurch, et
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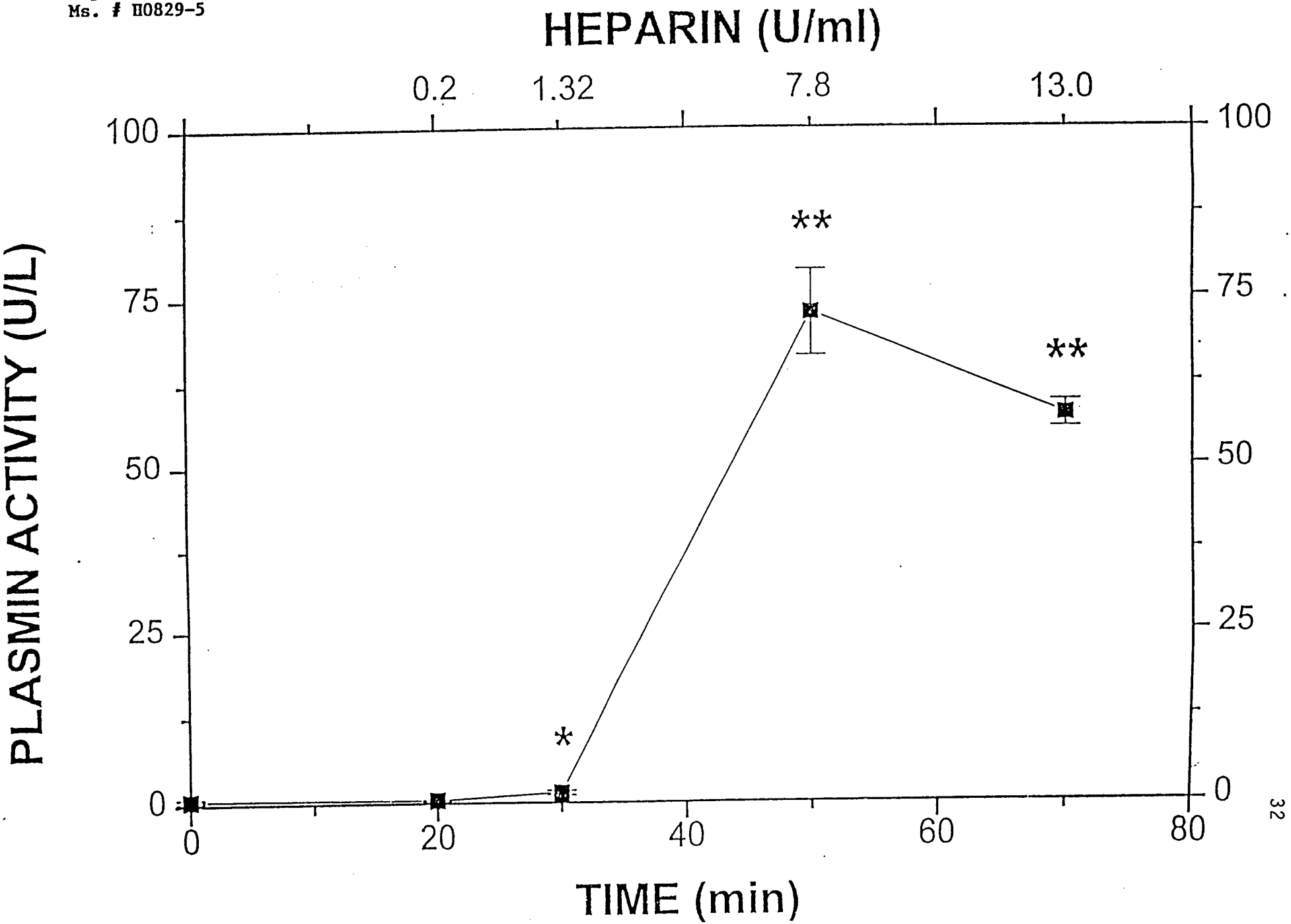

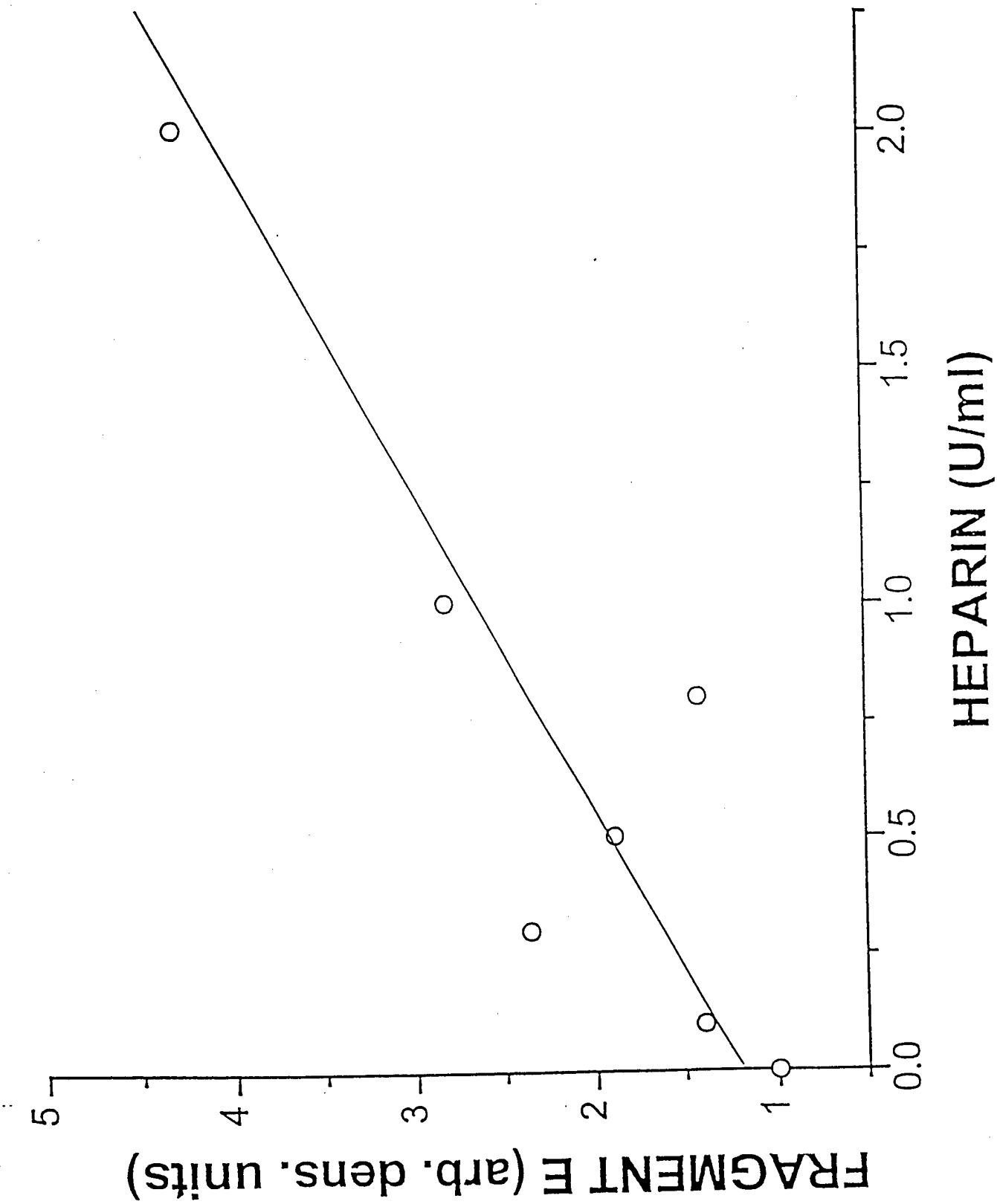

Figure 4, Upchurch, et al.
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Figure 5A, Upchurch, et al.
Ms. # H0829-5

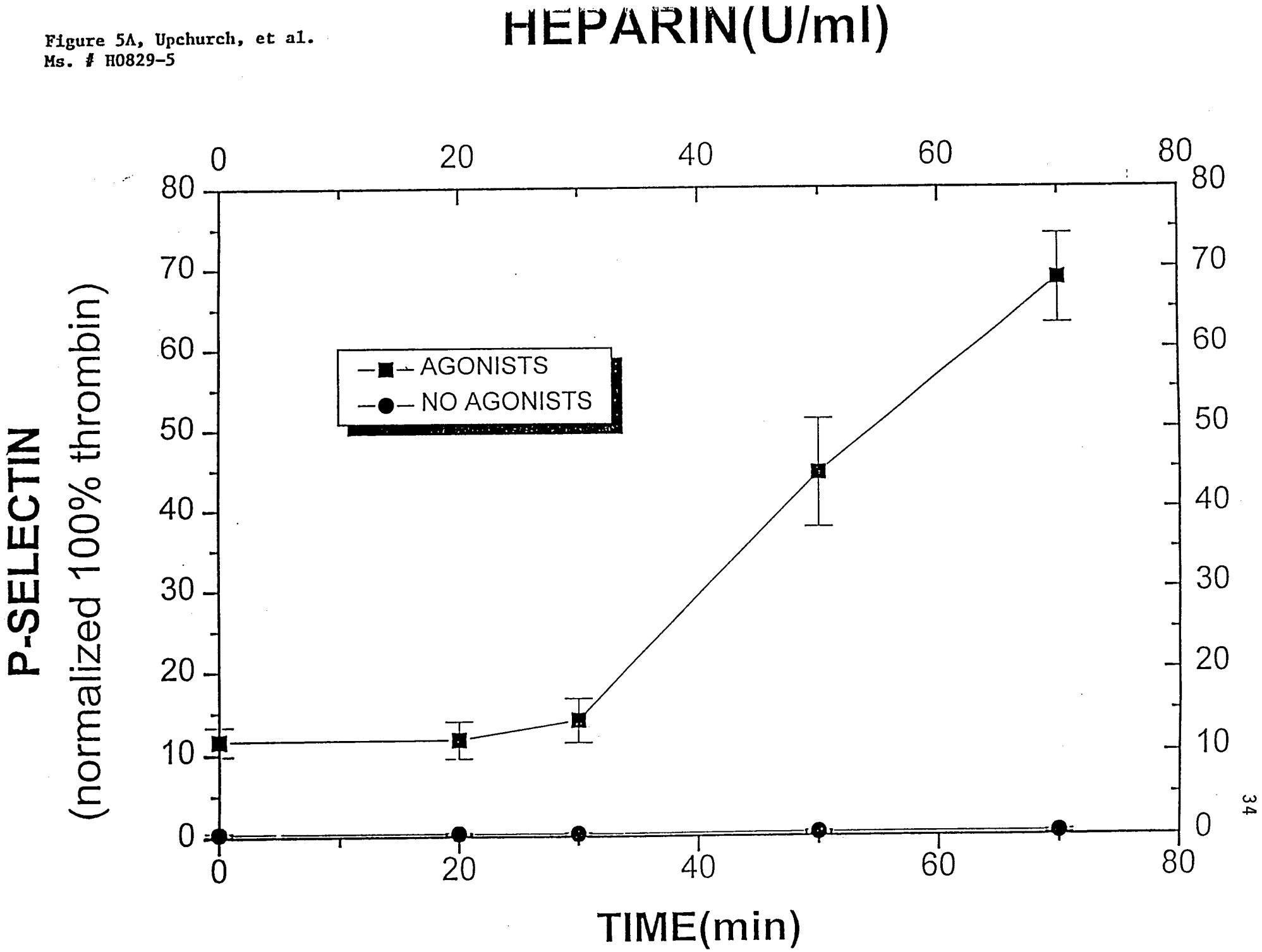

Figure 5B, Upchurch, et al.
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